EXHIBIT 13

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Characterization of human homologue of 4-1BB and its ligand

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1. Summary

The human homologue of 4-1BB (H4-1BB) cDNA was isolated from PMA plus ionomycin-treated human peripheral T-cell cDNA libraries. The amino acid sequence deduced from the nucleotide sequence showed that the protein is composed of 255 amino acids with 2 potential N-linked glycosylation sites. The molecular weight of its protein backbone is calculated to be 27 kDa. The H4-1BB contains features such as signal sequence and transmembrane domain, indicating that it is a receptor protein. This protein showed 60% identity of amino acid sequence to mouse 4-1BB. In the cytoplasmic domain there are 5 regions of amino acid sequences conserved from mouse to human, indicating that these residues might be important in the 4-1BB function. H4-1BB mRNA was detected in unstimulated peripheral blood T cells and was inducible in T-cell lines such as Jurkat and CEM. H4-1BB-AP, a fusion protein between the H4-1BB extracellular domain and alkaline phosphatase, was used to identify the ligand for the H4-1BB. Although the H4-1BB ligand was detected in both T and B cells of human peripheral blood, the ligand was preferentially expressed in primary B cells and B-cell lines. Daudi, a B-cell lymphoma, was one of the B-cell lines that carried a higher number of ligands. Scatchard analysis showed that the $K_d = 1.4 \times 10^9$ M and the number of ligands in Daudi cell was 4.2×10^3.

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2. Introduction

Murine 4-1BB was initially isolated from activated T-cell clone by a modified differential screening procedure [1]. 4-1BB mRNA was not detectable in resting splenocytes or unstimulated cloned T cells [2]. Activation of T cells by anti-CD3 (or anti-TCR$\alpha\beta$) mAb induced 4-1BB mRNA within 3 h of stimulation. The induction of 4-1BB mRNA was inhibited by cyclosporin A treatment [3]. The level of 4-1BB mRNA increased when the stimulation persisted and remained at a high level up to 6 days post-stimulation in vitro [2,3].

4-1BB is structurally related to the members of NGFR/TNFR superfamily, which are characterized by the presence of 3–6 patterns of a cysteine-rich motif in their extracellular domains. The NGFR/TNFR superfamily also includes low-affinity nerve growth factor receptor (NGFR), 2 receptors for tumor necrosis factor (TNFR-I and TNFR-II), CD30, CD40, OX40, Fas and CD27 [4]. These molecules appear to be involved in cell growth, survival and death processes. Although these receptors possess structurally similar extracellular domains, the cytoplasmic domains of these proteins are different, providing the means for diversity in transmembrane signaling. Recently, ligands for CD40, CD27, CD30, Fas and 4-1BB were identified and their cDNAs were cloned [5]. They are type II membrane proteins and homologous to TNF, fulfilling the expectation that when receptors are similar, the structure of their ligands may also be similar.

4-1BB exists as both a 30 kDa monomer and a 55 kDa dimer on T-cell surfaces [3]. Cross-linking of 4-1BB with anti-4-1BB mAb (1AH2) in the presence of anti-CD3 resulted in an up to 9.6-fold increase in T-cell

proliferation, compared to anti-CD3 stimulation alone on day 3 of T-cell culture [3]. 4-1BB signaling may be mediated by $p56^{lck}$ because 4-1BB is physically associated with $p56^{lck}$ [6]. Stimulation of 4-1BB ligand in mouse B cells also enhanced B-cell proliferation [7]. These results indicate that the 4-1BB system may produce a bidirectional signal. 4-1BB was induced in the intraepithelial lymphocytes (IEL) $\gamma\delta$ T cells, and its cross-linking enhanced proliferation of the IEL cells [8]. We have isolated a human homologue of the murine 4-1BB and have compared its properties with the mouse 4-1BB.

3. Materials and Methods

3.1. Design of PCR primers

The areas of sequence conservation among the TNFR/NGFR superfamily were chosen. Forward primer I (H4-1BBFI) spans from amino acids 36–41, and forward primer II (H4-1BBFII) spans from amino acids 52–58 of the mouse 4-1BB. Reverse primer (H4-1BBRI) spans from amino acids 116–121 and reverse primer II (H4-1BBII) spans from amino acids 122–128 of mouse 4-1BB. The degenerative sequences of PCR primers are as follows:

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H4-1BBFI: 5' TTC TGT CGI AAA TAT AAT CC 3'
            T   C A     G   C   C
H4-1BBFII: 5' TTC TCI TCI ATT GGI GGI CA 3'
               T  G   G   C
                          A
H4-1BBRI: 5' CC IAA IGA ACA IGT TTT ACA 3'
               G  CT G       C   G
H4-1BBRII: 5' TT TTG ATC ATT AAA IGT TCC 3'
              C   G   G   G
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3.2. Cloning of human 4-1BB cDNA

Peripheral blood lymphocytes from normal healthy individuals were isolated and activated with PMA (10 ng/ml) and ionomycin (1 μM). Using reverse transcriptase the human lymphocyte mRNA was converted to single-stranded cDNA. The cDNA was then amplified with Taq polymerase with the primers. The combination of primers was as follows: H4-1BBFI vs. H4-1BBRI; H4-1BBFI vs. H4-1BBRII; H4-1BBFII vs. H4-1BBRI; and H4-1BBFII vs. H4-1BBRII. An amplified cDNA fragment was used to screen the activated human T-cell cDNA library to obtain the full-length cDNA.

3.3. Production of H4-1BB-AP fusion protein

The 5′ portion of the H4-1BB cDNA including sequences encoding the original signal peptide and the entire extracellular domain, was amplified by polymerase chain reaction (PCR). For correctly oriented cloning, a *Bgl*II site at the 5′ end of the forward primers and a *Hind*III site at the 5′ end of the reverse primers were created. The *Bgl*II–*Hind*III H4-1BB fragment was inserted into a mammalian expression vector APtag-1, upstream of the coding sequence for human placental alkaline phosphatase (AP) [9]. Sequence analysis of the fusion region confirmed that the H4-1BB and AP sequences were joined in frame (data not shown). The 4-1BB-AP plasmid, linearized with *Cla*I, was co-transfected with the linearized selectable marker plasmid, pSV7neo, by the calcium phosphate co-precipitation method. After selection in 500 μg/ml G418, neomycin resistant colonies (Neo^r-NIH-3T3) were picked and expanded. Northern and Western analyses, and the AP assay were used to select for clones that produce high levels of H4-1BB–AP in the supernatant.

3.4. Alkaline phosphatase assay

Each sample was assayed for AP activity in triplicate. AP activity was measured by incubating 100 μl of heat-inactivated supernatants with 100 μl of 2 × AP buffer (1 × = 1 M diethanolamine, pH 9.8, 0.5 mM $MgCl_2$, 10 mM homoarginine, 0.5 mg/ml BSA and 12 mM *p*-nitrophenyl phosphate), and measuring the absorbance at 405 nm (A_{405}) [9]. When determining relative H4-1BB–AP or AP activity, serial dilutions were performed so that AP activity was measured at non-saturating levels. The H4-1BB–AP or AP was then diluted accordingly, so equivalent levels of H4-1BB–AP or AP activity were added to each sample.

3.5. Production of bacterial H4-1BB and anti-H4-1BB antiserum

The extracellular portion of the mature H4-1BB (sH4-1BB) was expressed in bacteria as a GST (glutathionine S transferase) fusion protein using PGEX-3 vector (Pharmacia). The sH4-1BB fraction was purified by a GST-Sepharose column and Sepharose 4B column chromatographies after the H4-1BB–GST fusion protein was cleaved with factor Xa. The recombinant H4-1BB was mixed with TiterMax (CytRX) and injected subcutaneously into a female NZW rabbit (Harlan, Indianapolis, IN). A second injection was performed 1 month after the first one. The antiserum was obtained 7 days after the second injection. IgG fraction of the antiserum was purified over a protein G affinity column.

3.6. Reagents

Phytohemagglutinin (PHA), phorbol-12-myristate-13-acetate (PMA), neuraminidase, and Histopaque-1077

were purchased from Sigma (St. Louis, MO). Pokeweed mitogen (PWM) was from Gibco, human IgM from Calbiochem, and anti-human CD3–FITC, anti-human CD19 and mouse IgG-FITC were from Olympus (Lake Success, NY).

3.7. Cells

Sheep red blood cells were obtained from Sigma. NIH-3T3 cells were maintained in DMEM containing 10% fetal bovine serum (FBS) supplemented with antibiotics. MT-4, CEM, Daudi, SKW 6.4, U937, Jurkat and EB3 cells were maintained in RPMI-1640 containing 10% FBS supplemented with 1 mM sodium pyruvate, 1 mM sodium glutamate, 50 μM 2-mercaptoethanol and antibiotics (RPMI-CM).

3.8. Isolation of T and B cells from peripheral blood

Human peripheral blood was purchased from the Central Indiana Regional Blood Center. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque gradient centrifugation. Monocytes/macrophages were depleted by incubating the PBMC in the petri dishes. The non-adherent lymphocytes were collected. T cells were obtained by rosetting procedure. The lymphocytes (10^7/ml) were layered on FBS and centrifuged at 800 rpm at 20°C for 5 min. The cell pellets were resuspended in 1 ml of RPMI-10, and mixed with 2 ml of FBS and 2 ml of neuraminidase-treated sheep RBC. The mixture was incubated at 37°C for 10 min, centrifuged at 800 rpm for 5 min and then incubated on ice for 1 h. The mixture was gently resuspended. The rosetted T cells and B cells were separated by Ficoll-Hypaque gradient centrifugation. This T-cell preparation was more than 95% $CD3^+$ and B-cell preparation was more than 70% $CD19^+$.

3.9. T- and B-cell activation

Human primary T cells were stimulated by PHA at 5 μg/ml and harvested at different time points. The primary B cells were stimulated with PWM in the presence of T cells for the first 3 days. After 3 days, the T cells were removed by using rosetting procedure and the B cells were continued to be stimulated with PWM and exogenous IL-2 for another 3 days. Human T-cell lines Jurkat and CEM were stimulated with PMA (10 μg/ml) and Ionomycin (1 μM), or PHA (10 μg).

3.10. Immunoblot analysis of H4-1BB-AP

Purified H4-1BB–AP was visualized by a standard immunoblotting procedure as described [3] with anti-H4-1BB antiserum and a secondary antibody against rabbit IgG (H + L)-alkaline phosphatase (Zymed).

3.11. H4-1BB–AP binding assay

Cells (2×10^6 cells for cell lines or 6×10^6 cells for primary lymphocytes) were washed with HBHA buffer (Hanks' balanced salt solution with 0.5 mg/ml BSA, 0.1% NaN_3, 20 mM Hepes (pH 7.3)). Samples in 100 μl were incubated in Eppendorf tubes with equivalent levels of H4-1BB–AP or AP activity for 60 min at 4°C with slow rotation. The cells were pelleted and washed 3 times with HBHA buffer. Cells were then lysed in 300 μl of 1% Triton X-100 and 10 mM Tris-HCl, pH 8.0. The lysate was vortexed vigorously and centrifuged at $10\,000 \times g$ for 5 min. Cell lysates were incubated at 65°C for 15 min to destroy the endogenous AP activity. AP activity was determined as above. To determine the specific binding of H4-1BB–AP, recombinant sH4-1BB was added at different concentrations to the assay mixture.

3.12. Northern blot analysis

Total RNA from human T-cell lines, or primary T cells were used to detect H4-1BB transcription by a standard Northern blot method as described [10].

3.13. FFCS analysis

Daudi, Jurkat cells, or stimulated or unstimulated primary T or B cells (2.5×10^5 cells/sample) were incubated in binding medium (1% BSA in HBSS, 0.1% NaN_3) alone or in binding medium containing 2 μg/ml H4-1BB–AP or AP for 30 min in an ice bath. Cells were washed twice with binding medium and then stained with FITC-conjugated mouse anti-human placental AP antibody (anti-AP) (Medix Biotech, Foster City, CA) for 30 min. The cells were washed twice with the binding buffer and analyzed by FACScan (Becton Dickinson).

4. Results and Discussion

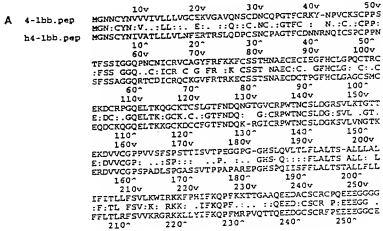
4.1. Human homologue of mouse 4-1BB (H4-1BB)

To isolate the human homologue of mouse 4-1BB (H4-1BB), two sets of polymerase chain reaction (PCR) primers were designed. In the design process, the amino acid sequence among the members of TNFR/NGFR superfamily were compared and areas of sequence conservation were chosen. The amino acid sequences employed were mouse 4-1BB, human tumor necrosis factor receptors, human CD40, and human CD27.

The primer set of H4-1BBFII and H4-1BBRII produced a specific band of ~240 bp, the expected size of human 4-1BB if the H4-1BB is similar to mouse 4-1BB in size. The PCR product (240 bp) was cloned in PGEM3 vector and sequenced. One open reading frame of the PCR product was ~65% identical to mouse 4-1BB. Therefore, it was tentatively concluded that the 240 bp PCR product represented a portion of H4-1BB. The 240 bp PCR product was used to screen a λgtII cDNA library of activated human T lymphocytes. An ~0.85 kb cDNA was isolated. The alignment of predicted amino acid sequence between human and mouse 4-1BB is shown in Fig. 1. There was approximately 60% amino acid homology between the two sequences. Since the cytoplasmic domain may contain features that may be involved in signal transduction, we examined the amino acid sequence motifs that were conserved between mouse and human in the cytoplasmic domain.

Five regions that were of potential significance were identified: (1) IFKQPF motif; (2) two consecutive threonines; (3) two runs of acidic amino acid sequence; (4) potential lck-binding domain and (5) consecutive glycines (Fig. 1B). Among them the threonines could be a potential site for phosphorylation. The potential lck-binding site was not completely conserved in the human. Therefore, whether H4-1BB associates with lck remains to be tested.

Schwartz et al. [11] published a human receptor cDNA called ILA. H4-1BB sequence was virtually identical to that of ILA. There were two nucleotide differences in the coding region between H4-1BB and ILA. The differences resulted in either conservative



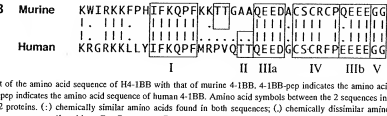


Fig. 1. A: alignment of the amino acid sequence of H4-1BB with that of murine 4-1BB. 4-1BB-pep indicates the amino acid sequence of murine 4-1BB and h4-1BB-pep indicates the amino acid sequence of human 4-1BB. Amino acid symbols between the 2 sequences indicate identical amino acids found in the 2 proteins. (·) chemically similar amino acids found in both sequences; (,) chemically dissimilar amino acids found in both sequences. The 2 sequences were aligned by a Bestfit program. B: cytoplasmic tails of mouse and human 4-1BB have conserved regions that may mediate signal transduction. Five clusters of sequence conservation between mouse and human include (I) IFKPPF motif, (II) 2 consecutive threonines; (IIIa and IIIb), 2 runs of acidic amino acid, (IV) potential lck-binding domain, and (V) consecutive glycines.

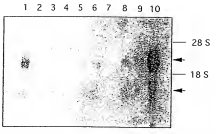


Fig. 2. H4-1BB mRNA expression. Total cytoplasmid RNA from the following cells were isolated and analyzed for 4-1BB mRNA expression by Northern analysis. Peripheral blood T lymphocytes were stimulated with medium alone (lane 1) or with PHA for 3 days (lane 2). Jurkat cells were stimulated with medium alone (lane 3) or with ionomycin and PMA for 3 h (lane 4), for 6 h (lane 5), or for 12 h (lane 6). CEM cells were stimulated with medium alone (lane 7), or with ionomycin and PMA for 3 h (lane 8), for 6 h (lane 9), or 16 h (lane 10).

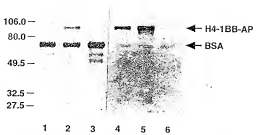


Fig. 3. Recombinant H4-1BB–AP fusion protein. Purified H4-1BB–AP fusion protein was analyzed by Coomassie Blue staining (lanes 1–3) and by a Western blot (lanes 4–6). 100 ng (lanes 1 and 4), 200 ng (lanes 2 and 5), or 0 ng (lanes 3 and 6) of the purified H4-1BB–AP were mixed with 3 μg of carrier protein BSA and run on a 10% SDS-PAGE. Lanes 1–3 were stained with Coomassie Blue and lanes 4–6 were probed with rabbit anti-H4-1BB serum. Molecular sizes are shown on the left.

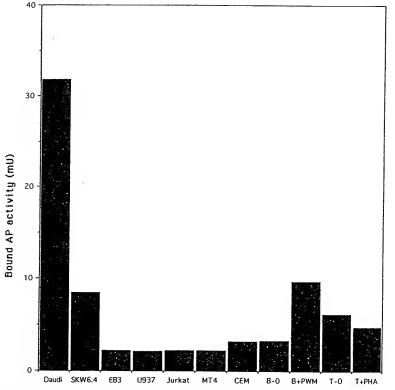


Fig. 4. Analysis of H4-1BB–AP binding to primary peripheral T and B cells, lymphoid and monocyte cell lines. Two million cells per sample were incubated with H4-1BB–AP or AP, washed and assayed for bound AP activity. Daudi, SKW 6.4 and EB3 are B-cell lymphoma cell lines. U937 is a monocytic cell line. Jurkat, MT4 and CEM are T cell lines. B-O and T-Q cells are unstimulated peripheral B cells and T cells, respectively. B+PWM is PWM-stimulated peripheral blood B cells and T+PHA is PHA-stimulated peripheral blood T cells.

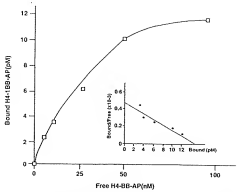


Fig. 5. Equilibrium saturation binding of H4-1BB–AP to Daudi cells. Daudi cells were incubated with increasing concentrations of H4-1BB–AP in the absence or presence of 100-fold excess of recombinant sH4-1BB. Bound AP activity was measured. All samples were measured in triplicate and the maximum standard deviation of any measurement was < 10%.

amino acid change; codon 107. AAA (Lys) → AGA (Arg), or resulted in no amino acid change; codon 163, GCT (Ala) → GCC (Ala).

We used the H4-1BB cDNA to detect H4-1BB mRNA expression in primary T cells and a variety of T cell lines. Unstimulated primary T cells produced H4-1BB mRNA. However, when the T cells were stimulated with PMA for 3 days, H4-1BB mRNA level was reduced. Whether the reduction of H4-1BB mRNA level in PMA-stimulated primary T cells was a result of cell death or of an actual down-regulation of transcription was not determined. Unstimulated Jurkat and CEM cells did not express the H4-1BB mRNA. However, when the cells were stimulated with PMA and ionomycin, H4-1BB mRNA was detected. The induction reached its peak at 16 h post-stimulation (Fig. 2). As in the case of murine 4-1BB [1], the H4-1BB gene produced two different sizes (2.4 kb and 1.4 kb) of transcript.

4.2. H4-1BB-AP fusion protein

The H4-1BB–AP-expressing plasmid and PSV7neo were cotransfected into NIH3T3 cells. A high H4-1BB–AP producer, H4-1BB–AP-31 was selected among the G418-resistant clones. H4-1BB–AP was purified by an affinity column chromatography with an anti-AP-conjugated Sepharose column, from H4-1BB–AP-31 culture supernatant. The purified H4-1BB–AP with carrier protein BSA was run on 10% SDS-PAGE (Fig. 3). The H4-1BB–AP was a 90 kDa protein, consisting of a 23 kDa extracellular portion of H4-1BB and 67 kDa AP protein (lanes 1 and 2), and was recognized specifically by anti-H4-1BB antibodies (lanes 4 and 5).

4.3. Analysis of H4-1BB–AP binding to lymphoid cell lines, and primary B and T cells

Measurement of AP activity provided a simple and reliable method for an initial determination of the relative amount of 4-1BB–AP bound to one cell type versus another cell type. Cells were incubated in 1 ml of HBSS containing equivalent levels of H4-1BB–AP or AP activity (12 U/ml). In all experiments, cell viability was ≥ 95% as determined by Trypan Blue exclusion. The cells were washed, lysed, and assayed for AP activity. All cells incubated with AP alone bound negligible levels of AP activity (data not shown).

The highest level of H4-1BB–AP binding was observed with the B-cell lymphoma Daudi (Fig. 4). Another B-cell line SKW6.4 showed a modest level of H4-1BB–AP binding. In contrast, U937 (human monocytic leukemia cell line) or T-cell lines showed a negli-

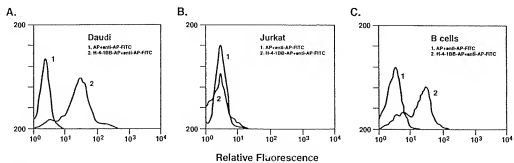


Fig. 6. FACS analysis of H4-1BB–AP binding. Daudi (A), Jurkat (B) or primary B cells (C) were incubated with binding medium containing AP or H4-1BB–AP and stained with anti-AP–FITC.

gible level of H4-1BB–AP-binding. Peripheral T and B cells were purified and examined for binding to H4-1BB–AP. As shown in Fig. 4 both T and B lymphocytes showed a low level of H4-1BB–AP binding, without any stimulation. When the primary B cells were stimulated with PWM for 4 days, H4-1BB–AP binding was elevated (Fig. 4). When the primary T cells were stimulated with PMA H4-1BB–AP binding tended to be decreased (Fig. 4). These results indicate that H4-1BB ligand preferentially expresses on B lymphocytes.

4.4. Characterization of the H4-1BB–AP binding sites

Purified H4-1BB–AP was utilized for a competition binding assay. A representative binding curve is shown in Fig. 5. Daudi cells expressed approximately 4200 binding sites/cell. The linear characteristics of Scatchard analysis predicted that Daudi cells expressed one binding site with a $K_d = 1.4 \times 10^9$ M. These results are very similar to those obtained from the mouse B-cell line 2PK3 cells [7].

4.5. FACS analysis

To determine the percentage of cells expressing the H4-1BB binding site, FACS analysis was performed using purified H4-1BB–AP and AP followed by a FITC-conjugated anti-AP antibody. In agreement with the binding assay, the Daudi cells expressed a high level of the H4-1BB–AP binding site, whereas the Jurkat cells did not bind to the H4-1BB–AP. Interestingly, unstimulated peripheral B cells also expressed 4-1BB–AP binding sites (Fig. 6).

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